Rapid and selective quantification of drug compounds for cassette-dosing DMPK studies by multi-MRM triple quadrupole MS

Rapid method development with the Agilent 1200 RRLC system and Agilent 6410 triple quadrupole LC/MS for compound quantification in DMPK studies

Application Note

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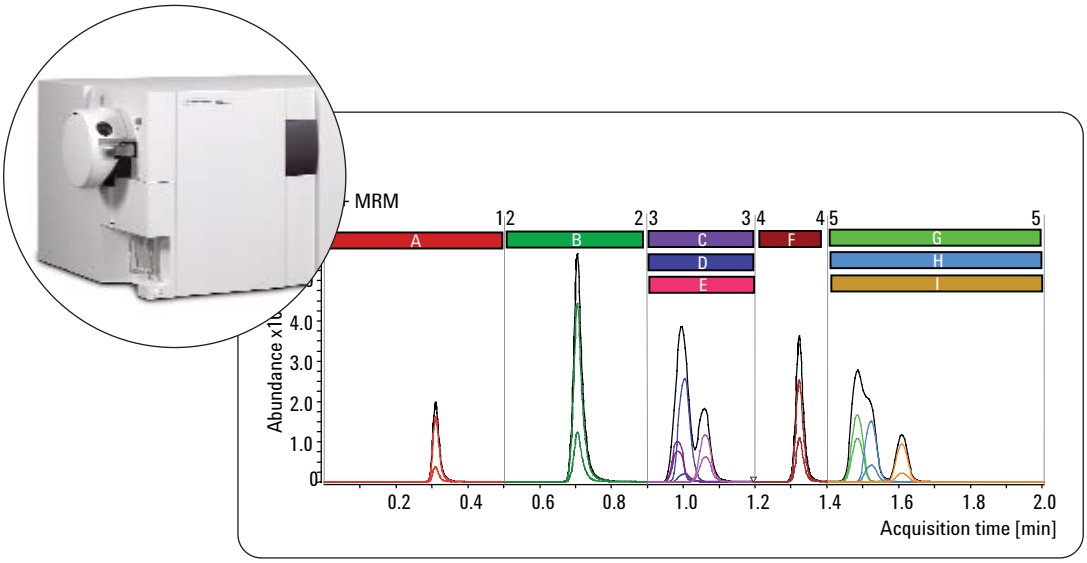

Agilent Equipment:

1200 Series RRLC
6410 triple quadrupole LC/MS
ZORBAX RRHT column
MassHunter workstation software

Application Area:

Quantification of drug compounds in DMPK studies
High throughput of DMPK samples

Abstract

This Application Note describes:

- The rapid development of an MRM triple quadrupole MS method for compound quantification.
- The fast liquid chromatography separation on 1.8 µm RRHT columns for high throughput of DMPK samples.
- The application of the developed LC/MS MRM method for the quantification of compounds in a mixture by a multi MRM method.

Agilent Technologies

Introduction

For successful drug discovery research today, it is essential to have rapid screening protocols for determining biological activities and pharmacokinetic parameters of possible new drug compounds[1]. The application of cassette dosing provides an approach for rapid screening of a large number of compounds for their in vivo pharmacokinetic properties[2]. The typical experimental protocol for a cassette dosing study involves simultaneous administration of 5-10 compounds from a similar chemical series to animals, for example, dogs[3]. The technique of tandem LC/MS has delivered the technical possibilities for rapid and simultaneous quantification of drugs in plasma samples obtained through cassette dosing experiments[4,5]. To make the concept of cassette dosing practicably applicable, it is essential that the triple quadrupole mass spectrometer is capable of fast and sensitive multi component quantification, achievable by utilisation of the MRM (multi-reaction monitoring) mode. In biological samples ion suppression caused by matrix ions is often a problem, which can be overcome by means of rapid resolution liquid chromatography (RRLC) to elute discrete compounds into the ion source of the mass spectrometer. In this Application Note we demonstrate the rapid development of a method with the Agilent 1200 RRLC system and Agilent 6410 triple quadrupole LC/MS for quantification that is necessary for cassette-dosing DMPK studies.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution (RR) LC system
- Agilent ZORBAX SB C-18, 2.1 x 50 mm, 1.8 µm particle size (rapid resolution high throughput (RRHT) column).
- Agilent 6410 triple quadrupole LC/MS system
- Agilent MassHunter Workstation for data acquisition and quantitative data analysis

LC/MSMS (MRM) method

- Agilent 1200 Series SL pump
  Solvent A: water + 0.1 % formic acid (FA)
  Solvent B: ACN + 0.1 % formic acid (FA)
  Flow: 1 mL/min
  Gradient: 10-45 %B in 2 min
  Stop time: 2 min
  Post time: 2 min

- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Thermostat temperature: 4 °C
  Needle wash: 5 s in wash port with methanol
  Sample flush out factor: 20
  Automated delay volume Reduction after injection and flushing by switching the injector loop to bypass

- Agilent 1200 Series SL column compartment
  Column temperature: 50 °C

- Agilent 1200 Series variable wavelength detector (VWD)
  Detection wavelength: 200 nm
  Peak width: 0.25 s
  Flow cell volume: 4 µL
  Flow cell path length: 3 mm

- Agilent 6410 triple quadrupole LC/MS system
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  Time segments: 0, 0.5, 0.9, 1.2 and 1.4 min.
  MRM settings for each time segment: Precursor ion mass –> quantifier ion mass
  Dwell time: 35 ms (it is possible to acquire up to 99 MRM transitions with a dwell time of 5 ms per time segment)
  Optimized fragmentor voltage: 140 V
  Individually optimized collision energy

Flow injection method for optimization of fragmentor voltage

- Agilent 1200 Series SL pump
  Flow: 0.5 mL/min
  Solvent: 65 %A (water + 0.1 % FA), 35 %B (ACN + 0.1 % FA)
  Stop time: 5 min

- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Sample temperature: 4 °C
  Injector program:
  Remote start pulse,
  Repeat 9 times,
  Valve bypass,
  Draw sample,
  Valve mainpass,
  Wait 0.25 min,
  End repeat.

- Agilent 6410 triple quadrupole LC/MS system
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  Time segments: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 min
  MS2 Scan mode: 100-400 m/z
  Scan time: 200 ms
  Fragmentor voltages according to the time segments: 60, 80, 100, 120, 140, 160, 180, 200 and 220 V

Flow injection method for optimization of collision energy

- Agilent 1200 Series SL pump
  Flow: 0.5 mL/min
  Solvent: 65 %A (Water + 0.1 % FA), 35 %B (ACN + 0.1 % FA)
  Stop time: 6 min

- Agilent 1200 Series SL autosampler
  Injection volume: 1 µL
  Sample temperature: 4 °C
  Injector program:
    Remote Start pulse
    Repeat 11 times
    Valve bypass
    Draw sample
    Valve mainpass
    Wait 0.25 min
    End repeat

- Agilent 6410 triple quadrupole LC/MS
  Source: ESI, positive
  Temperature: 300 °C
  Gas flow: 12 L/min
  Nebulizer pressure: 60 psi
  Capillary voltage: 4000 V
  Time segments: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 min
  Product Ion
  Scan mode: 100-400 m/z
  Scan time: 200 ms
  Optimized fragmentor voltage: 180 V
  Collision energy according to the time segments: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 V

Results and discussion

For complex samples, which contain chemically similar compounds with only unit differences in their molecular mass within a complex matrix, it is not sufficient to create only extracted ion chromatograms (EIC) for their identification and quantification because of the possible extraction of compounds with an isobaric mass. Therefore, for such experiments triple quadrupole mass spectrometers are used in MRM mode where the parent ion is isolated in the first quadrupole and after fragmentation in the collision cell a specific fragment is isolated. This transition from parent to fragment ion gives enough specificity and sensitivity for identification and quantification. To get the optimum performance in sensitivity it is necessary to adjust the fragmentor voltage of the instrument for highest signal intensity of the precursor ion and subsequent optimization of the collision energy gives the highest intensity for the main fragment. For a cassette-dosing experiment of 9 (A–I) new possible drug compounds a detection and quantification MRM method has to be developed. A solution of the compounds at a concentration of 1 µg/mL for each compound was prepared. For the optimization of the fragmentor voltage and collision energy the described flow injection methods with an injection of 1 ng on column (1 µL of the stock solution) were used. From the obtained total ion chromatogram (TIC) the masses of all compounds were extracted automatically and the data file was uploaded to the Agilent MassHunter qualitative data analysis software with a predefined EIC method (figure 1). Within the outlined time segments the fragmentor voltage was started at 60 V and increased in 20 V steps up to 220 V. After reviewing the EICs for all compounds, generated in the fast 5 minute flow injection analysis, the optimum fragmentor voltage for each compound was determined to be 140 V.

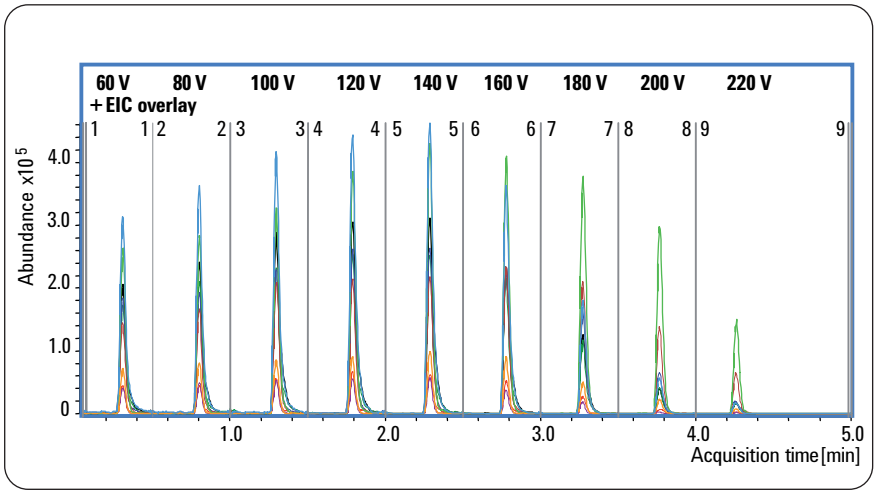

Figure 1
Optimization of fragmentor voltage for nine test compounds in parallel, showing overlay of nine EICs.

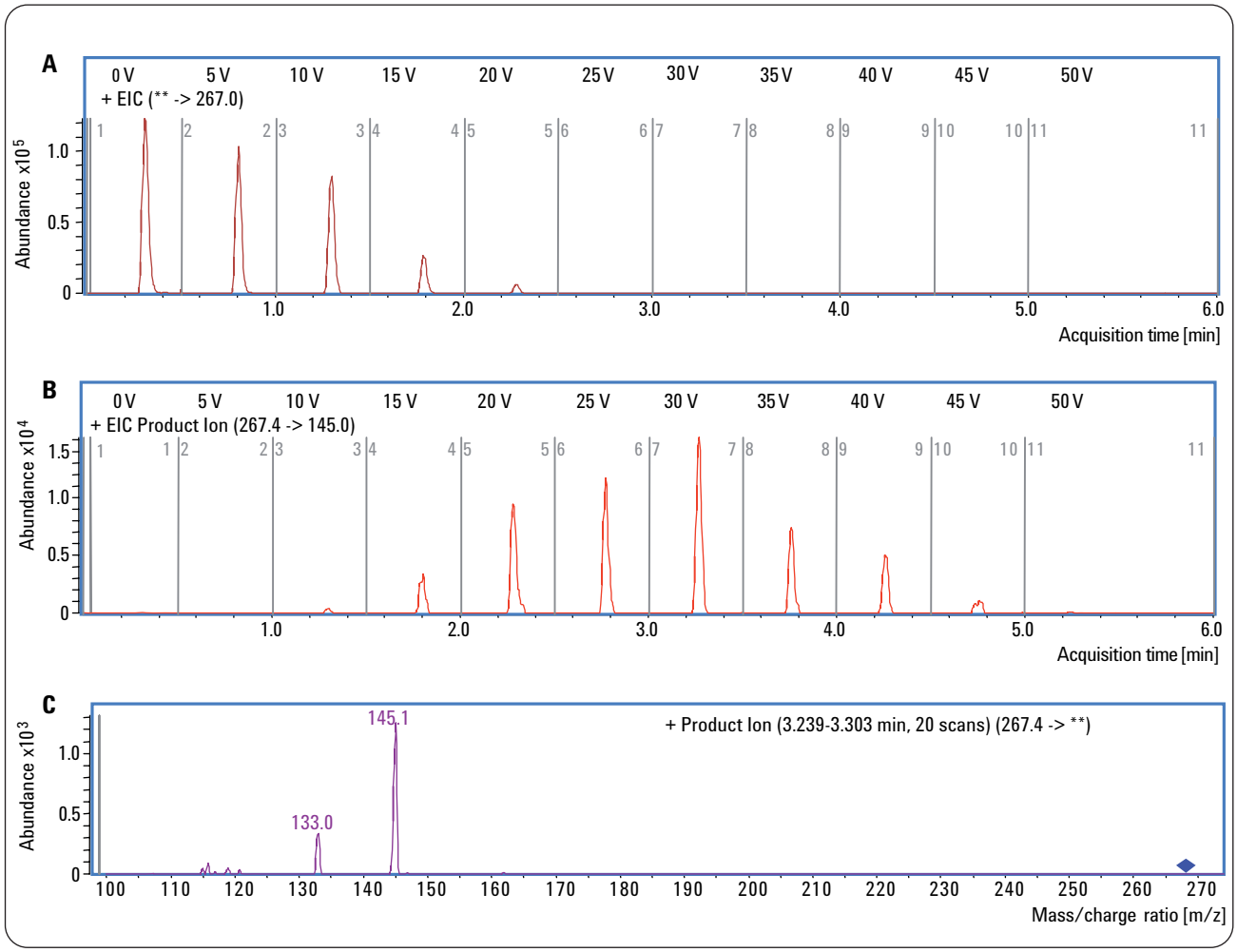

Figure 2
Collision energy optimization for compound G:
A) EIC of the precursor ion at m/z 267.4
B) EIC of the product ion at m/z 145.1
C) MS/MS spectrum of the precursor ion at m/z 267.4.

With this optimized fragmentor voltage an additional flow injection experiment was performed to optimize the collision energy for each compound fragmentation. Each subsequent injection time segment of the analysis was acquired with increasing collision energy in steps of 5 V between 0 and 50 V and using the product ion scan mode isolating the individual mass of interest (figure 2).

The EIC for the precursor mass of compound G shows clearly a decreasing signal with increasing collision energy caused by the fragmentation into the product ions (figure 2A). The emerging main fragment is seen at m/z 145.1. The extraction of the transition 267.4 to 145.1 shows an increasing signal for increasing collision energy with an optimum intensity at 30 V (figure 2B). The complete MS/MS spectrum for the fragmentation of the parent ion m/z 267.4 shows the intense fragment ion at m/z 145.1 and the less intense ion at m/z 133.0, which were chosen as the quantifier and qualifier ions respectively (Figure 2C). With the obtained optimized MS conditions MRM transitions for the chosen quantifier and qualifier ions were programmed within specific time segments according

to the retention time of each compound (figure 3). In the time segments 3 and 5, six MRM transitions were acquired for the transitions of the compounds C, D, E and G, H and I respectively.

To apply the developed method for the detection of the drug molecules it is important to know the limit of detection (LOD). To achieve this, a dilution series down to 0.1 pg on-column for each compound was measured (figure 4). For compound B a LOD of 100 fg (on-column) was determined for a signal-to-noise of 4.4. The LOD achievable with the used LC/MSMS method was easily able to meet the requirements for sensitivity. This is in range of 5 to 5000 ng/mL, for standard cassette-dosing experiments[5]. With this optimized method it is possible to gain quantification data for pharmacokinetic studies, by analysis of the MS data with the quantitative MassHunter software.

For real quantitative analysis of the data it was necessary to set up a series of calibration concentrations, which were used after measurement for quantification with the quantitative MassHunter software. To create the necessary calibration curves for all compounds in the cassette a series of concen-

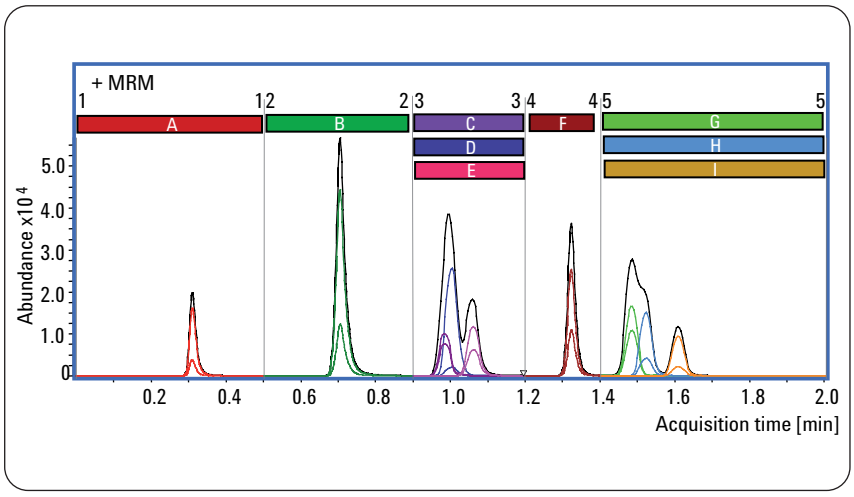

Figure 3
Multi-MRM method for nine compounds (A-I). Special transitions for quantifier and qualifier ions were set up in time segments according to the retention time of each compound.

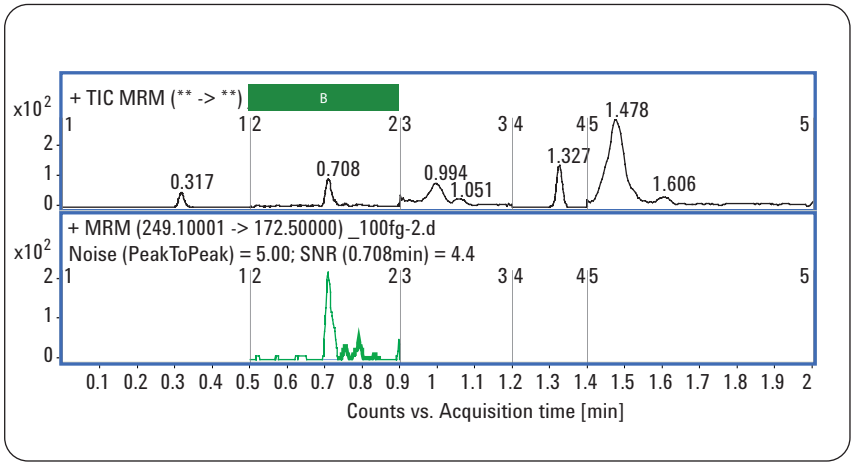

Figure 4
Measurement of a sample with a concentration of 100 fg on column for each compound and determination of the limit of detection at S\N = 4.4 for compound B. (noise measured 0.73-0.85 min, bold region).

trations (10, 5, 2, 1, 0.5, 0.2 pg on-column for each compound in mixture) was measured. After loading the data in the quantitative MassHunter software, a method for quantification was created. The method contained all masses for quantifiers and qualifiers as well as concentration levels, and created the calibration curve (figure 5 and 6). The calibration curve showed good linearity over six levels between 0.2 and 10 pg on-column with R^2 = 0.995 (figure 5). The qualifier at m/z 145.1 as well as the quantifier at m/z 133.0 and their ratio were used to support the result by using the ratio level as quality criterion for the quantification of the correct compound automatically in the software (figure 6).

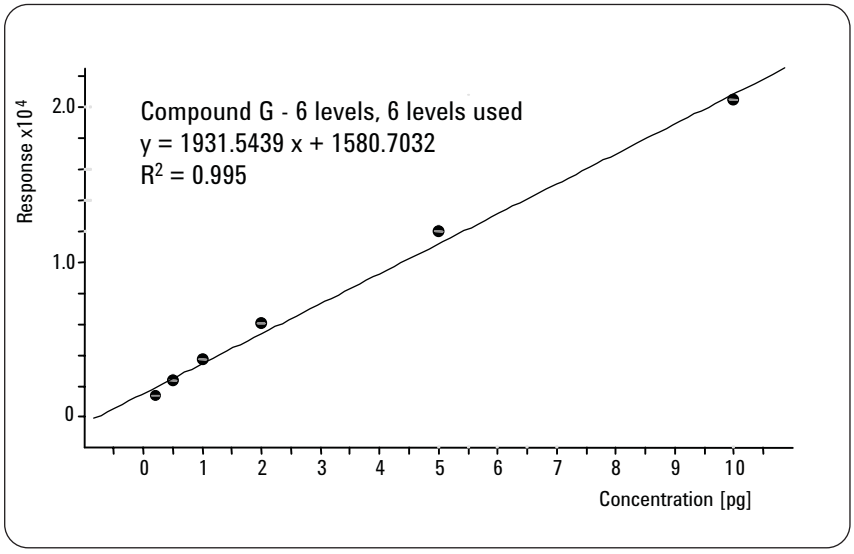

Figure 5
Calibration curve for compound G over six levels (10, 5, 2, 1, 0.5, 0.2 pg) with linearity R2 = 0.995.

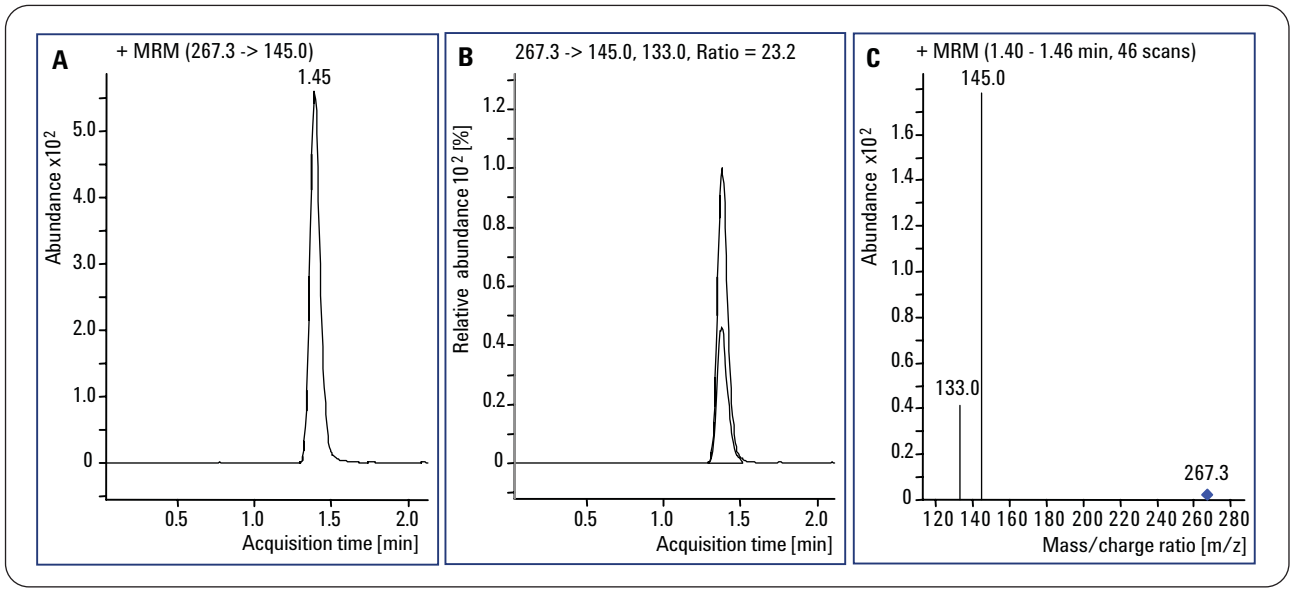

Figure 6
Example MRM graphic results for quantification of compound G:
A) EIC of the quantifier transition of compound G
B) Overlayed EICs of the quantifier and qualifier transitions showing their ratio
C) Combined MS/MS spectrum of compound G

Conclusion

This Application Note demonstrates the rapid development of a MRM triple quadrupole MRM LC/MSMS method for a group of new drug candidates prior to a DMPK cassette-dosing experiment. By means of flow injection analysis of a mixture of the compounds, the fragmentor voltage and collision energy for MRM experiments under optimized conditions were determined within a few minutes. With this optimized method the limit of detection for the compounds was determined to be in the femtogram range. For an absolute quantification experiment a low concentration calibration curve with good linearity was performed. The results obtained demonstrated the usability of the Agilent 6410 triple quadrupole together with the Agilent 1200 Series RRLC system for rapid MRM method development for new compounds in DMPK studies.

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